

APE1 overexpression in XRCC1-deficient cells complements the defective repair of oxidative single strand breaks but increases genomic instability

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ABSTRACT

XRCC1 protein is essential for mammalian viability and is required for the efficient repair of single strand breaks (SSBs) and damaged bases in DNA. XRCC1-deficient cells are genetically unstable and sensitive to DNA damaging agents. XRCC1 has no known enzymatic activity and is thought to act as a scaffold protein for both SSB and base excision repair activities. To further define the defects leading to genetic instability in XRCC1-deficient cells, we overexpressed the AP endonuclease APE1, shown previously to interact with and be stimulated by XRCC1. Here, we report that the overexpression of APE1 can compensate for the impaired capability of XRCC1-deficient cells to repair SSBs induced by oxidative DNA damage, both *in vivo* and in whole-cell extracts. We show that, for this kind of damage, the repair of blocked DNA ends is rate limiting and can be performed by APE1. Conversely, APE1 overproduction resulted in a 3-fold increase in the sensitivity of XRCC1-deficient cells to an alkylating agent, most probably due to the accumulation of SSBs. Finally, the overproduction of APE1 results in increases of 40% in the frequency of micronuclei and 33% in sister chromatid exchanges of XRCC1⁻ cells. These data suggest that the spontaneous generation of AP sites could be at the origin of the SSBs responsible for the spontaneous genetic instability characteristic of XRCC1-deficient cells.

INTRODUCTION

Thousands of single strand breaks (SSBs) in DNA are formed each day in a mammalian cell. They can arise directly from reactions with endogenously generated reactive oxygen species (ROS), or indirectly as intermediates in the repair of damaged bases or abasic (AP) sites. It has been estimated that the majority of the several thousand AP sites generated per mammalian cell per day by both spontaneous depurination and in the course of base excision repair (BER) reactions, are readily cleaved and converted into SSBs (1). If not repaired, SSBs cause genomic instability leading to increased mutation rates and chromosomal rearrangements (2). In the case of their induction by genotoxic challenges, they result in a reduction in cell survival.

The Chinese hamster ovary (CHO) mutant EM9 cell line was originally isolated by its hypersensitivity to alkylating agents and is shown to be cross-sensitive to ionizing radiation (3). EM9 cells display a high level of spontaneous or induced sister chromatid exchanges (SCEs) (4) and a slower rate of SSB repair (SSBR) when compared with the parental AA8 cell line (3). All these phenotypes are due to the inactivation of the *XRCC1* gene (5). Although no enzymatic activity has been ascribed to XRCC1, its absence leads to a substantial reduction in the levels of its partner Ligase 3 (Lig3) (6). However, the destabilization of Lig3 cannot account for all the phenotypes that result from the inactivation of XRCC1 (7). The finding of multiple interactions of XRCC1 with other DNA repair enzymes, such as DNA polymerase β (Pol β) (8,9), poly (ADP-ribose) polymerases 1 and 2 (PARP 1 and 2) (8,10,11), polynucleotide kinase (PNK) (12), AP endonuclease 1 (APE1, also known as HAP1 or REF1) (13) and more recently, a DNA glycosylase (14) and the proliferating cell nuclear antigen (PCNA) (15), lead to the proposal that XRCC1 acts

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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as a scaffold protein that coordinates the enzymatic steps in both BER and SSBR (13–16). The lack of coordination in the repair process could result in the persistence and exposure to the cellular milieu of potentially toxic DNA intermediates such as SSBs.

Frequently SSBs, and in particular those generated by ROS, carry chemical modifications at the 3' and/or 5'-DNA termini that impede further gap filling or religation. Among the most common of such modifications encountered on the 3'-terminus are 3'-phosphate (3'-P), 3'-phosphoglycolate (3'-PG) or 3'-open aldehydes (3'-dRP). The 3'-dRP extremities are also potentially formed as repair intermediates by the so-called bi-functional DNA glycosylases during BER of oxidized bases, although the *in vivo* relevance of the 3'-dRP BER intermediate is not known (17,18). Processing of the 3'-blocked ends is therefore required to complete repair. In mammalian cells, several activities have been proposed to 'clean' 3'-termini (12,19). However, it is clear that the main activity capable of removing 3'-dRP is provided by APE1 (20). Biochemical experiments using whole-cell extract (WCE) showed that, in spite of its relative inefficiency *in vitro*, APE1 is also the rate-limiting activity for the removal of 3'-PG generated by the oxidation of AP sites or direct breaks (21,22). As for the 3'-P resulting from either direct breaks by ROS or the activity of DNA glycosylases such as Neill1 or Neill2 (23), two enzymes are known to process them *in vitro*, APE1 and PNK. Indeed, APE1, in addition to cleaving 5' of an AP site, can also hydrolyze 3'-P (20). Similarly, PNK displays a 3'-phosphatase activity in addition to acting as a kinase (12,24). Interestingly, both enzymes, APE1 and PNK, not only interact with XRCC1 but also have their 3'-phosphatase activity stimulated by this protein *in vitro* (12,13). Stable down-regulation of hPNK by small-interfering RNA sensitizes cells to genotoxic agents (25). Similarly, cells with reduced levels of APE1, obtained by inactivation of one allele of the mouse *Apex* gene (26), by antisense expression of the APE1 cDNA (27,28) or by siRNA expression (29) in human cell lines, are hypersensitive to a wide range of oxidative genotoxic agents. As these phenotypes, likely to result from a deficient repair of 3'-blocked DNA ends generated by ROS, are similar to those obtained in XRCC1-deficient cells, some of the *xrcc1*-/- phenotypes could result from the reduced capacity of these cells to 'clean' 3'-blocked ends (12). Here, by over-expressing APE1 in EM9 cells and characterizing the resulting cellular and biochemical phenotypes, we analyse some of the potential mechanisms by which XRCC1 protects cells against the consequences of DNA damage and explore the type of spontaneous lesion that is most likely to contribute to the genetic instability of XRCC1-deficient cells.

MATERIALS AND METHODS

Cell lines and culture conditions

CHO cell lines AA8 and EM9 (5) were obtained from E. Sage (Institut Curie, Paris). The XRCC1-complemented EM9-XH cell line (30) was kindly provided by K. W. Caldecott (University of Sussex). All the mammalian cell lines were grown in DMEM containing sodium pyruvate and supplemented with 10% foetal calf serum, 1% penicillin/streptomycin and 1% glutamine at 37°C in a 5% CO₂ humidified atmosphere.

Overproduction of APE1 in CHO cell lines

In order to overproduce hAPE1 in EM9 or parental AA8 cells, *hAPE1* gene open reading frame was amplified by PCR using the primers 5'-CCGGAATTCATGCCGAAGCGTGGAAA-AAGG and 5'-GCTCTAGACAGTGCTAG GTA TAGGT and inserted in the pcDNA3.1/myc-His A vector (Invitrogen) between EcoRI and XbaI restriction sites. The resulting plasmid, pPR311, allows the expression of hAPE1 in frame with a Myc epitope and a polyhistidine tag, under the control of the cytomegalovirus promoter. Twenty four hours after transfection, cells were plated on 100 mm dishes with complete medium containing 800 µg/ml of geneticin. Single clones were isolated after 15 days and amplified.

For APE1 expression analysis, a total of 8×10^6 cells was resuspended in 0.2 ml of lysis buffer [20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 250 mM NaCl and 0.4 mM AEBSF (ICN Biomedicals Inc.)] containing 0.8 µg/ml of antipain, 0.8 µg/ml of aprotinin and 0.8 mg/ml of leupeptin, sonicated for 8 s (1 s pulse every 10 s) and centrifuged at 4°C for 1 h at 13 000 r.p.m. The supernatant was recovered and its protein concentration was determined using the Bio-Rad Protein Assay kit (Bio-Rad). For the detection of hAPE1 by western blotting, 50 µg of WCE was separated by denaturing 12.5% PAGE and transferred onto a nitrocellulose membrane (Hybond-C; Amersham). A rabbit polyclonal anti-APE1 antibody (gift of I. D. Hickson, University of Oxford) diluted to 1:1000 was used to detect APE1.

APE1 cleavage assay

To test the enzymatic activity of hAPE1, a 34mer oligonucleotide containing a tetrahydrofuranyl residue (F) (Eurogentec) at position 16, was labelled at the 5' end using [γ -³²P]ATP (3000 Ci/mmol; Amersham) and T4 PNK (New England Biolabs). The ³²P-labelled strand was hybridized with the complementary oligonucleotide carrying a cytosine (C) opposite F by incubation at 90°C for 10 min followed by slow cooling down to room temperature. In a standard reaction (16 µl final volume), 50 fmol of the labelled duplex was incubated in reaction buffer (25 mM Tris-HCl, pH 8.0, 1 mM MgCl₂ and 0.4 mg/ml of BSA) with 100 ng of WCE at 37°C for 45 min. The reaction was stopped by addition of 6 µl of formamide dye and heating at 95°C for 5 min. Products were resolved by denaturing 20% PAGE in the presence of 7 M urea. Gels were scanned and band intensities quantified using a Storm PhosphorImager (Molecular Dynamics).

Survival curves

Cultures at 80% confluence were trypsinized and 100–800 cells were plated in 6-well plates. After incubation for 16 h, cells were treated with methyl methanesulfonate (MMS) (Sigma) for 1 h at 37°C in complete medium or with H₂O₂ (Sigma) for 20 min at 37°C in phosphate-buffered saline (PBS). After treatment, the cells were washed with PBS. Fresh culture medium was added and the cells were incubated for 8 days to allow clones to grow. Clones were washed twice with PBS, fixed in a solution containing 20% acetic acid and 80% ethanol and stained with a 4% Giemsa solution (Reactifs RAL) for 2 h. After washing with water, visible colonies were counted. Each survival curve was carried out at least three times for statistical evaluation.

Induction and *in vivo* repair of DNA damage

For the induction of SSBs, cells were either treated with *tert*-butyl-hydroperoxide (75 μ M) or H₂O₂ (75 μ M) in DMEM without supplements for 15 min at 37°C and 5% CO₂. To induce AP sites and SSBs, the cells were incubated with 100 μ M MMS in DMEM without supplements for 30 min at 37°C and 5% CO₂. The cells were washed twice and then incubated at 37°C under culture conditions for the indicated repair times before DNA damage analysis.

Quantification of DNA modifications by alkaline elution

The alkaline elution assay originally described by Kohn *et al.* (31) with the modifications described previously (32,33) was used for the quantification of SSB and T4 endonuclease V-sensitive AP sites. Elution curves obtained with γ -irradiated cells were used for calibration, assuming that 6 Gy generate 1 SSB/106 bp (31). The numbers of DNA modifications observed in untreated control cells (background levels) were subtracted.

In vitro repair assays

To test the phosphatase activity of WCE, 100 fmol of a ³²P-labelled 34mer oligonucleotide containing an 8-oxoguanine residue (8-oxoG) at position 16 (gift of J. Cadet, CEA) and hybridized with its complementary oligonucleotide carrying a cytosine (C) opposite 8-oxoG was incubated with 5 ng of purified Fpg protein in a reaction mixture containing 20 mM Tris-HCl, pH 7.6, 1 mM DTT, 1 mM EDTA and 50 mM NaCl for 30 min. The resulting substrate, a 15mer 5'-labelled with ³²P carrying a one nucleotide gap with a 3'-P, was incubated in a reaction mixture containing 20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM DTT and 10 mM MgCl₂ in the presence of WCE. The amounts of extract and the incubation times are indicated in the legends to Figures 3 and 4. The reactions were stopped by adding 6 μ l of formamide dye and heating at 95°C for 5 min. The products were resolved by denaturing 20% PAGE in the presence of 7 M urea. Gels were scanned and band intensities quantified using a Storm PhosphorImager (Molecular Dynamics).

To evaluate the overall repair, reactions were carried out as described for the phosphatase assay but with the addition of 100 μ M dGTP, 2 mM ATP, 25 mM phosphocreatine (di-Tris salt; Sigma) and 2.5 μ g of creatine phosphokinase (type I; Sigma).

Determination of micronuclei

Trypsinized cells were resuspended in full culture medium and $\sim 1 \times 10^5$ cells were fixed on a microscope slide by cytospin centrifugation and treatment with methanol at -20°C for 1 h. After staining with bisbenzimidazole (Hoechst 33258) in Ca²⁺- and Mg²⁺-free PBS, 2000 cells were analysed for the presence of micronuclei with a fluorescence microscope (Nikon, Japan).

Sister chromatid exchange assay

A total of 6×10^5 cells were plated in a 25 cm² flask with medium containing 6 μ g/ml of BrdU. After two cell divisions in the presence of BrdU (30 h), Colcemid (Sigma) was added at a final concentration of 0.1 μ g/ml, for 2 h. Cells were then

washed with PBS, trypsinized, resuspended and centrifuged. The resulting pellet was resuspended in 0.075 M KCl and incubated for 15 min at 37°C. Before centrifugation, 100 μ l of the fixative (methanol-acetic acid, 3:1) is added. The pellet was resuspended in the fixative and incubated overnight at 4°C. The cells were centrifuged, washed in a fresh fixative, and dropped onto wet slides. The slides were submerged in 10 μ g/ml of Hoechst 33258 (Sigma) for 20 min, incubated for 2 h under UV light in a solution containing 0.3 M NaCl and 30 mM sodium citrate at pH 6.3 (SSC 2 \times , pH 6.3). The slides were plunged in SSC 2 \times , pH 6.3 at 60°C for 15 min and stained with 1.5% Giemsa for 3–5 min.

RESULTS

Overproduction of APE1 in CHO cell lines

In order to overproduce the APE1 protein in XRCC1-deficient EM9 or parental AA8 cells, the open reading frame coding the hAPE1 protein was cloned in frame with a *myc* tag and under the control of a constitutive promoter into a mammalian expression vector. After transfection, geneticin-resistant clones were tested for the overexpression of APE1 by western blotting. Independent clones were further selected by an enhanced AP site cleavage activity of their WCEs. One APE1 overproducing clone in each background (EM9-APE1 and AA8-APE1) was selected and characterized further. In parallel, EM9 and AA8 cell lines were transfected with the empty vector, to generate control cell lines EM9-V and AA8-V, respectively. Figure 1 shows the characterization of the WCE from the selected clones by western blotting, using an antibody against APE1 and by cleavage activity assay on a DNA substrate harbouring an AP site analogue. For the overproducers, a new band is revealed by the APE1 antibody, corresponding to the tagged protein expressed from the vector (Figure 1A). The expression of the exogenous protein in the

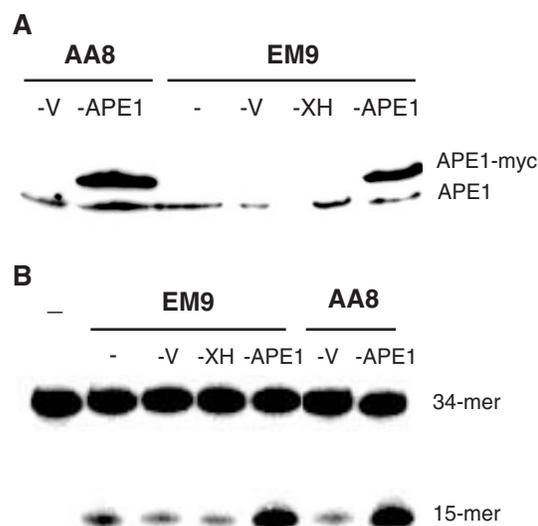


Figure 1. Overexpression of APE1 in CHO cell lines. (A) WCEs of transfected clones were analysed by western blotting using an anti-APE1 antibody. (B) APE activity of the WCEs (100 ng of total protein from each cell line) was measured by the cleavage of a 34mer duplex oligonucleotide carrying a tetrahydrofuran residue at position 16 of the 5'-³²P-labelled strand.

selected clones is associated with at least a 3-fold increase in the AP site-cleavage activity level (Figure 1B).

Acceleration of the repair of oxidative SSBs by APE1 overproduction in EM9 cells

EM9 cell lines were shown to be hypersensitive to a large spectrum of genotoxic agents known to generate SSBs (7). Among them, the agents causing an oxidative stress have widely been used to study the XRCC1-deficient cell lines (4,34). The hypersensitivity of the XRCC1-deficient cells to oxidative agents is generally associated with their reduced capacity to repair SSBs. To examine whether APE1 overproduction could alleviate this phenotype, we treated different EM9 cell lines with oxidants known to induce SSBs, and measured the *in vivo* SSBR kinetics using the alkaline elution assay. As expected, the XRCC1 deficiency in EM9 or EM9-V cells resulted in a reduced repair rate of SSB induced by *tert*-butyl-hydroperoxide when compared with EM9-XH cells corrected by XRCC1 (Figure 2A). Expression of APE1 in EM9-APE1 cell line resulted in a significant recovery of the

SSBR (Figure 2A). The repair kinetics for EM9-APE1 is intermediate between that of the XRCC1-deficient EM9 cells and that of the XRCC1-complemented EM9-XH cells. Similar results were obtained when *tert*-butyl-hydroperoxide was replaced by H₂O₂ as the damaging agent. When the cells were allowed to repair for 10 min after exposure to 75 μ M H₂O₂, the levels of remaining SSBs in the APE1 overproducer were again intermediate between those of EM9-V and EM9-XH (Figure 2B). Taken together, the above experiments show that the overproduction of APE1 partially compensates for the deficiency of XRCC1⁻ for repairing SSBs induced by ROS and that, in XRCC1-deficient cells, APE1 is able to accelerate a rate-limiting step of the repair of ROS-induced SSB.

As outlined in Introduction, SSBs termini induced by oxidative attack to DNA are frequently damaged and need to be processed to restore 3'-OH and 5'-P moieties to allow gap filling and DNA ligation (Figure 3A). Since this can be a limiting step in SSBR (16), we investigated the possibility that overexpressing APE1 in the XRCC1-deficient EM9 cells could accelerate the repair by WCEs of a gap with 3'-P, a common terminus modification found after DNA damage. We first compared the capacity of WCEs to mediate the removal of a 3'-P from a DNA substrate harbouring a gap with a 3'-P. EM9-V cell extracts were less able to remove the 3'-P, than the extracts from EM9-XH, reflecting a deficiency of the XRCC1⁻ cells in their 3'-phosphatase activity (Figure 3B, cf. lanes 2–5 and 6–9). When extracts from the APE1 overproducing cell line were tested, they displayed an even greater 3'-phosphatase activity than the one from the XRCC1-complemented cell line. Kinetics experiments confirmed that EM9-APE1 cell extracts repair ('clean') 3'-P termini faster than the XRCC1 complemented EM9-XH extracts (Figure 3C). These data demonstrate that an overexpression of APE1 in EM9 cells can compensate for their deficiency in the repair of a 3'-blocked end.

As the ligation step of SSBR (Figure 3A) in XRCC1-deficient cells is known to be impaired, we wished to examine whether the removal of the 3'-P was a limiting step in the overall repair by WCE of our gap substrate by adding to the reaction mixture, dGTP and ATP, and therefore allowing the nucleotide incorporation and ligation steps to proceed (Figure 3A). As expected, the overall repair of a 3'-P gap by WCE was slower in extracts from EM9-V than in those from EM9-XH (Figure 4A and C). Surprisingly, the expression of APE1 in EM9 cells was able to restore the overall repair to levels even higher than those of EM9-XH (Figure 4A, 34mer band and C). The accumulation of the intermediate DNA resulting from the incorporation of one nucleotide (Figure 4A and B) indicates that APE1 overexpression cannot circumvent the ligase deficiency. However, the increased availability of the polymerase substrate (3'-OH gap), shown in Figure 3B and C, allowed the EM9-APE1 WCE to reach an overall repair rate higher than that of EM9-XH extracts (Figure 4A). This is reflected in Figure 4C, where the final DNA product (34mer) arising from the gap filling and ligation reactions in the various cell extracts is plotted as a function of the incubation time. From the experiments with WCE, we conclude that in *xrcc1*^{-/-} cells, at least in the case of a 3'-P, the removal of the 3'-blocking lesion is a rate-limiting step in SSBR.

While the exposure to increasing concentrations of H₂O₂ or *tert*-butyl-hydroperoxide confirmed the hypersensitivity to oxidizing agents of EM9-V cells when compared to EM9-XH

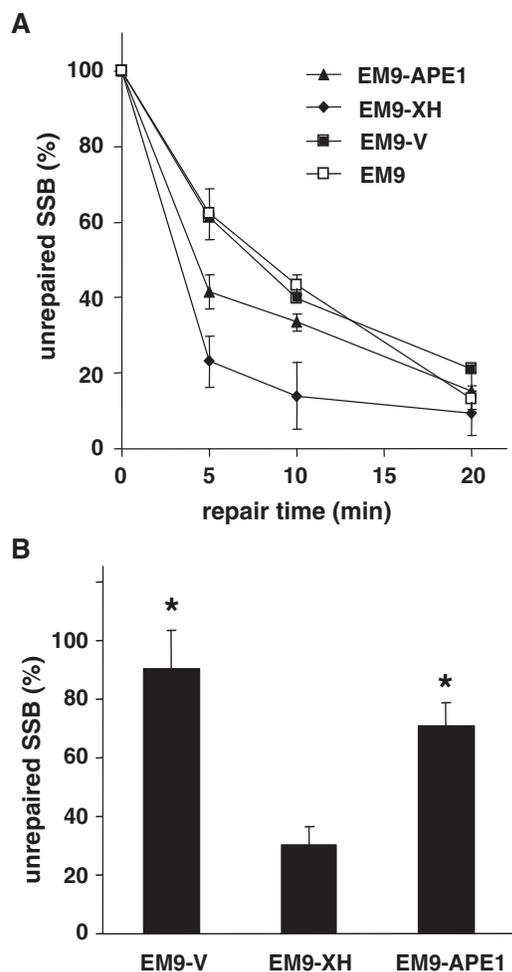


Figure 2. *In vivo* repair of ROS-induced SSBs induced by oxidative stress. (A) Residual SSBs were quantified by the alkaline elution method at different times after treatment of the cells with 75 μ M *tert*-butyl-hydroperoxide. The values given are the mean of three independent experiments \pm SD. (B) Residual SSBs were quantified 10 min after the end of treatment with 75 μ M H₂O₂ for 15 min at 37°C. Values are the mean of at least three independent experiments \pm SD. **P* < 0.02.

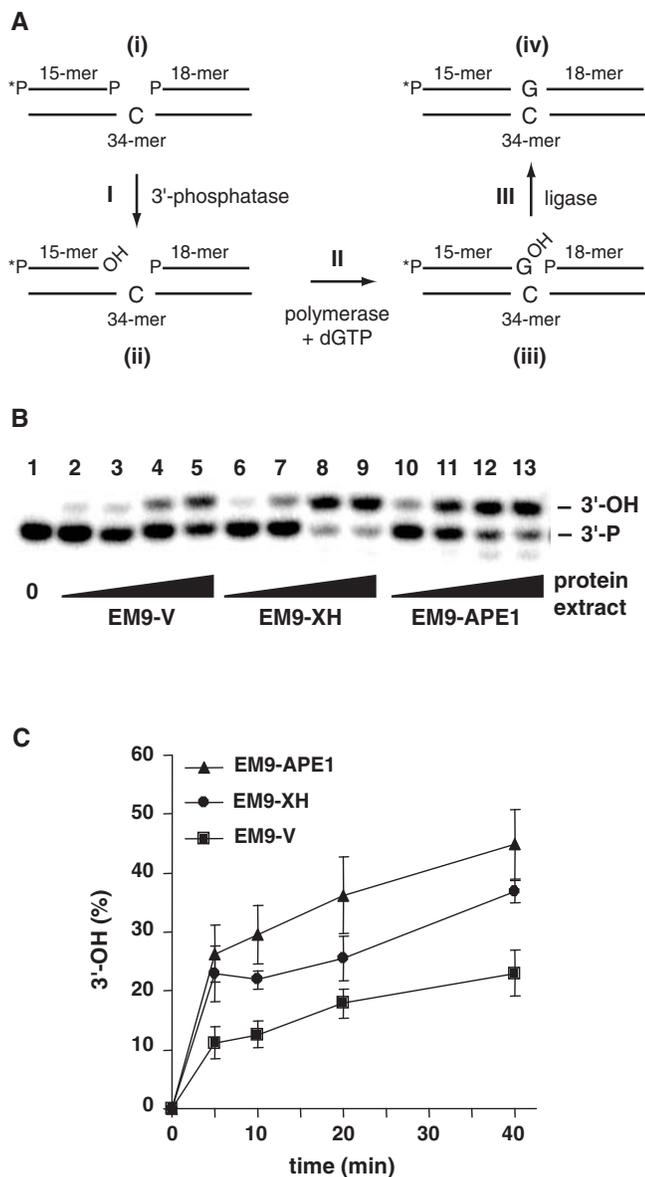


Figure 3. Phosphate removal from a 3'-P gap. (A) Schematic representation of the enzymatic reactions involved in the repair of a 3'-P harbouring gap. Initial substrate is a gap surrounded by 5'-P and 3'-P DNA ends (i). The 3' phosphate terminus is converted into a 3'-OH terminus by a 3' phosphatase (I) resulting in a gap (ii) that can be filled by a DNA polymerase (II, iii) and ligated (III) to yield a 34mer (iv). (B) The ^{32}P -labelled substrate (i) was incubated at 37°C for 15 min with 0.5 (lanes 2, 6 and 10), 1 (lanes 3, 7 and 11), 1.5 (lanes 4, 8 and 12) or 3 μg (lanes 5, 9 and 13) of WCE proteins from the indicated cell lines. Products were separated by denaturing PAGE. (C) 3'-Phosphatase gap substrate (i) was incubated with 1 μg of WCE of the indicated cell lines at 37°C and aliquots of each reaction were removed at the times indicated. The resulting products were separated by denaturing PAGE and the conversion of the 3'-P into 3'-OH was quantified.

expressing XRCC1, increasing cellular levels of APE1 does not improve the resistance of EM9 cells to H_2O_2 or *tert*-butylhydroperoxide (data not shown).

Accumulation of SSBs in APE1-overexpressing cells treated with an alkylating agent

Alkylating agents such as MMS generate high levels of AP sites, both by spontaneous depurination and in the course of

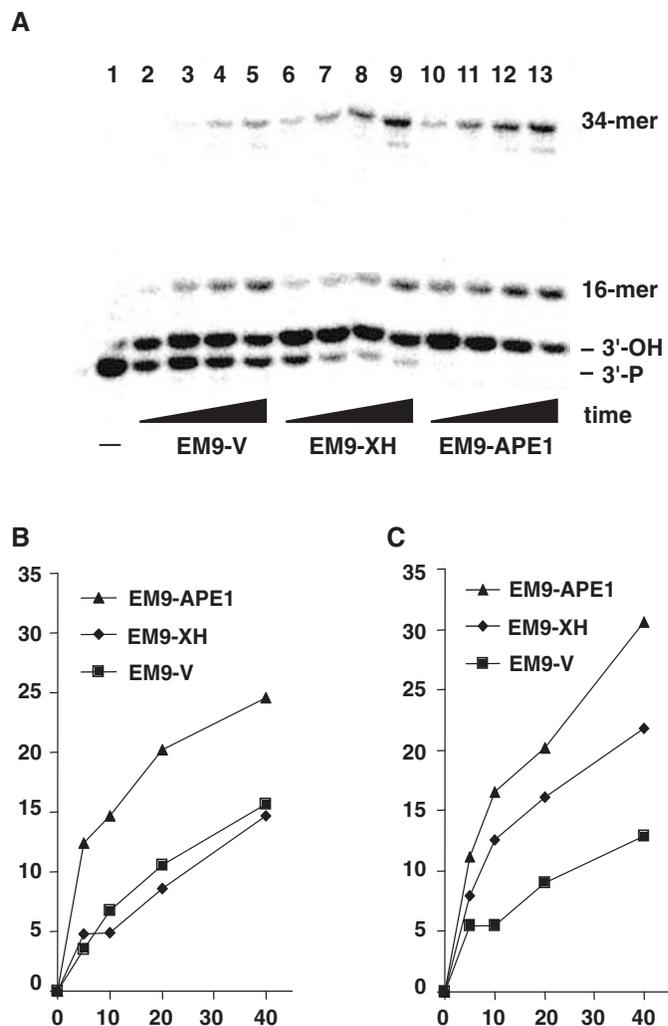


Figure 4. Overall repair of a 3'-P gap. The initial gap substrate shown in Figure 3A (i) was incubated at 37°C with 1 μg of protein from WCE from the indicated cell lines in the presence of ATP and dGTP. Aliquots of each reaction were removed at 5 (lanes 2, 6 and 10), 10 (lanes 3, 7 and 11), 20 (lanes 4, 8 and 12) and 40 min (lanes 5, 9 and 13). The products of the reaction were analysed by denaturing 20% PAGE (A). Quantification of the gel shown in (A) showing the percentage of 16mer (B) or 34mer (C) corresponding to the nucleotide incorporation and ligation products, respectively.

BER of alkylated DNA bases. The repair defect in EM9 cells is known to be associated with a pronounced hypersensitivity to alkylating agents and increased accumulation of SSB after treatment (3). We therefore wished to examine whether the defective processing of AP sites generated by MMS in EM9 cells could be modified by the overexpression of APE1. We compared the accumulation of AP sites and SSBs 0 and 15 min after exposure of the various cell lines to 100 μM MMS for 30 min at 37°C (Figure 5A). When compared with the XRCC1 complemented cells (EM9-XH), EM9-V cells showed higher levels of SSBs at both times analysed. The AP site levels in EM9-V cells were also elevated at the end of the treatment but after 15 min of recovery they were comparable to the levels in EM9-XH cells. These results show that the AP sites induced by the treatment are processed more slowly in the XRCC1-deficient cells and that SSBs accumulate during the recovery

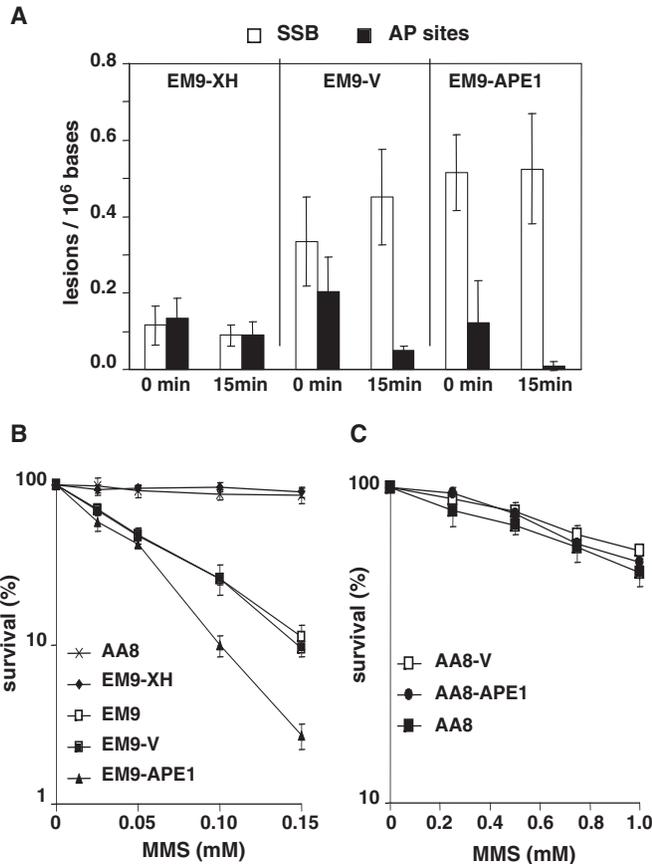


Figure 5. Responses to MMS. (A) Levels of SSBs and AP sites induced by treating various CHO cell lines with 100 μ M MMS for 30 min at 37°C as determined by alkaline elution at the end of the treatment (0 min) or after 15 min of recovery. (B and C) Effect of APE1 overproduction on survival to MMS treatment. Cells were treated with MMS for 1 h at 37°C at the indicated doses. Surviving clones (>50 cells) were scored after 8 days. Values represent the mean of at least three independent experiments \pm SD.

period. As expected, the overexpression of APE1 in EM9 cells resulted in decreased levels of AP sites when compared to those in EM9-V cells. However, the SSBs accumulated in the APE1-overproducing cells at higher levels than in the EM9-V cells. These results suggest that by overproducing APE1 in an XRCC1-deficient background, the cells readily cleave the induced AP sites, generating high levels of SSBs that cannot be processed efficiently owing to a downstream rate-limiting step. Further support for the negative consequences of an imbalance in the proteins responsible for the repair of AP sites emerged from the observation that EM9-APE1 cells exhibited an increased sensitivity to MMS, compared with the EM9-V cells (Figure 5B). In contrast, as it was previously shown (35–38), the overproduction of APE1 did not affect the sensitivity of the AA8 (XRCC1-proficient) cells to MMS even at very high concentrations of the genotoxic agent (Figure 5C).

The results from the repair of MMS-induced damage, along with the MMS sensitivity curves show that in XRCC1-defective cells, in contrast to the situation observed for the repair of ROS-induced SSBs, the rate-limiting step in the repair of normal abasic sites is downstream of the generation of the 3'-OH harbouring SSBs by the APE1.

Table 1. Micronuclei frequencies are enhanced in EM9 cells by overproduction of APE1

Cell lines	Micronuclei/2000 nuclei Mean	SD
EM9-XH	45	4
EM9-V	65	9
EM9-APE1	91*	14

SD, standard deviation.

* $P < 0.005$ (Student's *t*-test) for the difference between EM9-V and EM9-APE1.

Table 2. APE1 overexpression increases the level of sister chromatid exchanges

Cell lines	SCEs/cell Mean	SEM
EM9-XH	6.9	0.4
EM9-V	45.5	1.4
EM9-APE1	60.6*	1.8

SEM, standard error of the mean.

* $P < 0.0001$ (Mann-Whitney test) for the difference between EM9-V and EM9-APE1.

Overexpression of APE1 in EM9 cells increases spontaneous genomic instability

One of the landmark phenotypes of XRCC1-defective cells is their high level of SCE when grown in the presence of BrdU for one cell cycle (3,39). EM9 cells also display an elevated number of spontaneous chromosomal breaks when compared with the parental XRCC1-proficient cells. Both phenotypes have been attributed to a deficient repair of DNA lesions resulting in increased levels of SSBs (2,7). The results presented above show that the overproduction of APE1 in EM9 cells had opposite effects on the repair of DNA depending on the type of lesion present on DNA. Therefore, an analysis of the effect of overproducing APE1 in EM9 cells on the genetic stability in otherwise untreated cells should indicate which type of endogenous lesion contributes most to the genetic instability of untreated EM9 cells. Towards this purpose, we compared the number of micronuclei in the different EM9 cell lines used above. Table 1 shows that APE1 overproduction resulted in a 40% increase in micronuclei levels, a significant difference with the already elevated ones found in EM9 cells. Spontaneous SCE numbers (Table 2) also show a significant increase (33%) in the APE1 overproducers with respect to the number found for EM9-V, which is almost 7-fold higher than that of EM9-XH cells. These data suggest that the SSBs resulting from cleavage of endogenously generated AP sites could be an important cause of the genetic instability displayed by XRCC1-defective cells, while the SSBs generated by endogenously produced ROS appear to be less relevant.

DISCUSSION

XRCC1 and repair of AP sites

The 5'-dRP removal by Pol β is a rate-limiting step for single-nucleotide BER (40,41) in mammalian cells. Because XRCC1 has been shown to interact both physically and functionally

with APE1, Pol β and Lig3 (42), MMS hypersensitivity of EM9 cells could potentially be due to a deficiency in any of the enzymatic steps involved in the repair of the induced AP sites. Previous studies had shown that, in XRCC1-proficient cells, overproduction of APE1 did not affect the sensitivity of the cells to MMS (35–38). The surprising 3-fold increase in sensitivity of the EM9 cells to MMS by the overexpression of APE1, along with the increased accumulation of SSBs after treatment suggests that the more efficient processing of AP sites results in cleaved DNA intermediates that cannot be processed efficiently by the later steps of BER (or SSBR) due to the lack of XRCC1. This suggests that either the Pol β reactions or, more likely, the ligation step are limiting for the repair of normal abasic sites in XRCC1-deficient cells. The lack of coordination between the different enzymatic steps resulting from the absence of XRCC1 could also contribute to the hypersensitivity of EM9 cells to alkylating agents. By holding intermediate DNA substrates and handing them over to the next enzyme in the pathway, XRCC1 prevents potentially toxic BER or SSBR intermediates (single-stranded DNA, unligatable nicks, etc.) from being exposed to the cellular milieu (7,43). Therefore, accelerating the cleavage of AP sites without a concomitant increase in the downstream repair activities can lead to an excess of SSBs that cannot be processed correctly before triggering other reactions compromising cell viability.

Role of XRCC1 in oxidative SSBR

A very different scenario is observed for oxidative DNA damage processing in EM9 cells. APE1 overexpression could, in part, compensate for the defective repair kinetics of SSBs induced by oxidative agents such as H₂O₂ or *tert*-butyl-hydroperoxide (Figure 2). The experiments presented in Figures 3 and 4 indicate that the APE1 overexpression accelerates SSBR in EM9 cells by improving the processing ('cleaning') of blocked 3'-termini. The repair deficiency of EM9 cells at this step could be, in part, owing to a lack of activation of either PNK or APE1. Although it has been proposed that PNK is the enzyme responsible for the removal of 3'-P in cell extracts (24), our experiments show that APE1 can also perform the reaction to yield a 3'-OH and allow repair to be completed. Interestingly, APE1 has also been shown to provide the main activity for the removal of another 3'-blocking lesion, 3-PG (22). Taken together, these results suggest the involvement of APE1 in SSBR.

The experiments presented here also shed some light on the rate-limiting step in the repair of oxidative lesions. In the case of oxidative DNA damage leading to 3'-blocked ends, the removal of the blocking lesion is the limiting activity in XRCC1-deficient cells. Overexpressing APE1 could therefore relieve the slow repair phenotype by accelerating the removal of 3'-blocks. Consistently, HeLa cells display an increase in APE1 mRNA and protein when exposed to ROS (44), suggesting a mechanism to accelerate a rate-limiting step. The partial nature of the correction of SSBs displayed in Figure 2 is not surprising since 3'-blocked SSBs are only a subset of lethal lesions induced by those treatments. Genotoxic lesions other than 3'-blocked ends can also explain the lack of significant complementation of EM9 sensitivity to H₂O₂. Moreover, this lack of protection at the survival level does not exclude the possibility that the overproduction of APE1, by accelerating

SSBR and hence shortening the persistence of SSBs, could protect EM9 cells from other deleterious consequences of oxidative DNA damage, such as mutations or chromosomal rearrangements.

APE1 levels, XRCC1 and genetic instability

In BER, the balanced levels of the different proteins carrying out the enzymatic steps are a requisite to modulate their respective activities and to avoid the accumulation of potentially toxic repair intermediates (45). As shown here, overexpression of a repair activity can have opposite effects depending on the kind of lesion to be taken care of. In the case of APE1 overproduction in EM9 cells, this expression can accelerate a potentially rate-limiting step in the repair of ROS-induced DNA damage. It was indeed shown that heterozygosity for the mouse APE1 results in phenotypes associated with oxidative stress (26). Conversely, the consequences of MMS-induced damage seem to be exacerbated by the overexpression of APE1 in EM9 cells leading to enhanced sensitivity. This is unlike in XRCC1-proficient cells, where increasing the APE1 levels does not affect the sensitivity phenotypes (35–38). Interestingly, the increased expression of APE1 has been reported in several kinds of tumours (29,46–48), suggesting that the imbalance created by this overexpression of a BER enzyme can lead to genetic instability, as it is the case for Pol β overexpression (49).

The experiments presented above could also provide some hints on the kind of spontaneous lesions at the origin of the genetic instability in XRCC1-deficient cells. Indeed, the overproduction of APE1 in an *xrcc1*^{-/-} background leads to an increased number of spontaneous SCE as well as micronuclei. Since the overexpression of APE1 in EM9 cells accelerates the repair of SSBs generated by ROS (Figure 2) while having negative consequences on the processing of normal AP sites, it is tempting to speculate that SSBs arising from the processing of endogenously generated AP sites are more relevant for leading to the chromosomal instability characteristic of XRCC1-deficient cells than oxidized SSBs resulting from endogenous ROS generation.

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